

# ***U.S. PATENT APPLICATION***

***Inventor(s):*** Hiroshi TAMURA  
Y.C. LEE  
Isamu TAKAGAHARA  
Yuhsi MATUO  
Keiko SAITO  
Kichitaro KAWAGUCHI

***Invention:*** METHOD FOR DETERMINING CRP USING PHOSPHORYLCHOLINE

***NIXON & VANDERHYE P.C.  
ATTORNEYS AT LAW  
1100 NORTH GLEBE ROAD  
8<sup>TH</sup> FLOOR  
ARLINGTON, VIRGINIA 22201-4714  
(703) 816-4000  
Facsimile (703) 816-4100***

## ***SPECIFICATION***

## SPECIFICATION

### METHOD FOR DETERMINING CRP USING PHOSPHORYLCHOLINE

This application is a continuation of international application PCT/JP00/01268 filed March 3, 2000.

### TECHNICAL FIELD

5 The present invention relates to a novel method for determining levels of C-reactive protein (referred to as "CRP" hereinafter). More specifically, the invention relates to a novel method for effecting micro-quantitative analysis of CRP, using labeled phosphorylcholine (referred to as "PC" hereinafter).

### BACKGROUND OF THE INVENTION

CRP is a protein which is reactive with C-polysaccharide in the capsule of Diplococcus pneumoniae, and is present in the  $\beta$ -globulin fraction in serum. It consists of 5 identical peptides, each of which has a molecular weight of about 130 kDa. The concentration of CRP in the blood of a healthy human is below about 350 ng/ml, and dramatically increases to as much as 0.1 mg/ml during acute phase reactions such as occur during infection, inflammation or with tissue damage. CRP is thus recognized as an acute phase protein [Agrawal, A., Kilpartrick, J. M., and Volanakis, J. E. (1994) in Acute Phase Proteins-Molecular Biology, Biochemistry and Clinical Applications-(Mackiewicz, A., Kushner, I., and Baumann, H., eds.), pp. 79-92, CRC Press, London]. As a result of its characteristics, as mentioned above, CRP has been conventionally used as a diagnostic marker indicating

bacterial infection, inflammation and for post-surgery prognosis.

Although the biological role of CRP has not yet been completely elucidated, it has gradually been ascertained  
5 that CRP exerts various pro-inflammatory host-protection activities including complement activation [Richards, R. L., et al., (1997) Proc. Natl. Acad. Sci. USA 74, 5672-5676; Richards, R. L., et al., (1979) J. Immunol. 122, 1185-1189; Miyazawa, K., and Inoue, K. (1990) J. Immunol.  
10 145, 650-654; Jiang, H., et al., (1991) J. Immunol. 146, 2324-2330; Wolbink, G. J., et al., (1996) J. Immunol. 157, 473-479] and lymphocyte opsonization [Mortensen, R. F., et al., (1976) J. Immunol. 117, 774; Edwards, W. M., et al., (1982) J. Immunol. 128, 2498; Szalai, A. J., et al.,  
15 (1995) J. Immunol. 155, 2557-2563]. It is likely that these activities make contributions to non-specific host protection in the pre-immune phase of an inflammatory reaction.

In order to diagnose bacterial infection,  
20 inflammation and to assist in post-surgery prognosis, it is necessary for an acute phase protein level to be assayed accurately not only in an acute phase but also for it to be assayed accurately when a person is in a healthy state. CRP is an acute phase protein most sensitive and  
25 most highly reactive to extraneous stimuli [Morley, J. J., and Kushner, L. (1982) Ann. N. Y. Acad. Sci. 389, 406-418]. Therefore, the ability to accurately assay CRP levels in a healthy individual, will enable the early

stages of infection to be diagnosed more accurately. The early increase of CRP level at acute phase essentially needs to be detected in a rapid and accurate manner in order to monitor therapeutic treatment and to diagnose prognosis, wherein the detection requires a highly sensitive method capable of assaying serum CRP level within the normal range.

It is known that using a nephelometric immunoassay method [Yamamoto, S., et al., (1994) Vet. Quarterly 16, 74-77; Yamamoto, S., et al., (1993) Vet. Immunol. Immunopathol. 36, 257-264] or latex agglutination test [Sarikaputi, M., et al., (1992) Jap. J. Vet. Res. 40, 1-12; Yamada, T., et al., (1993) Ann. Clin. Biochem. 30, 72-76] CRP levels can be determined within a range of 1 to 1000 µg CRP/ml and within a range of 10 to 400 µg CRP/ml, respectively, in a simple manner. These ranges include the typical serum range (10 to 100 µg CRP/ml) in an acute phase. Since blood CRP concentration in a healthy individual is below about 350 ng/ml as stated above, however, the aforementioned methods are incapable of assaying CRP concentrations at within a normal range. Although these methods have been used widely at clinical tests, they are not sufficiently sensitive to detect early CRP level elevation at the beginning of an acute phase. Thus, assay results are simply expressed as "negative", "positive" or "elevation". In addition to the assay of CRP level at peak in acute phase, precise assay of normal CRP level or CRP level at the onset of acute phase is

extremely useful so as to enhance the precision of diagnosis.

Compared with the aforementioned methods, fluorescent immunoassay (FIA) [Siboo, R., and Kulisek, E. A. (1976) J. Immunol. Methods 23, 59-67] and radioimmunoassay [Claus, D. R., et al., (1976) J. Lab. Clin. Med. 87, 120-128] are considerably more sensitive. However, they are not sufficiently sensitive to effect assay of serum CRP in a healthy individual. Enzyme-linked immunosorbent assay (ELISA) [Lamb, W. R., et al., (1991) Med. Lab. Sci. 48, 201-205; Shields, M., et al., (1991) J. Immunol. Methods 141, 253-261; Yamamoto, S., et al., (1994) Vet. Quarterly 16, 74/77] is based on the same principle as those employed in both FIA and RIA, and represents a development of these methods. ELISA is a quantitative assay method, generally having greater sensitivity than that of RIA. When CRP concentration is assayed, however, no significant difference is found between the CRP range assayed by ELISA and the range assayed by RIA. Specifically, compared with the range of 8 to 800 ng CRP/ml as detected by RIA [Claus, D. R., et al., (1976) J. Lab. Clin. Med. 87, 120-128], the range detected by ELISA is 10 to 100 ng CRP/ml [Unten, S. K., and Hokama, Y. (1987) J. Clin. Lab. Anal. 1, 136-139]. These results are indicative of the fact that ELISA for CRP assay needs to be greatly modified.

#### SUMMARY OF THE INVENTION

The purpose of the present invention is to provide a method for rapidly determining CRP in a sample which

includes not only a CRP concentration in the serum occurring in an acute phase, but also serum CRP which is within the normal range, with a high degree of sensitivity.

5           Another purpose of the present invention is to develop a method for rapidly assaying CRP in a sample with a high degree of sensitivity and which receives no influence from frompentraxin family proteins having high homology to CRP present in serum, using phosphorylcholine  
10 (PC), and wherein the method is applicable in clinical diagnosis.

#### BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1 shows a method for determining CRP using  $\text{Eu}^{3+}$ -labeled probe, wherein (A), (B) and (C) show the results  
15 of the assay of CRP concentration by an antibody-antibody method, antibody-PC method and PC-antibody method, respectively;

Fig. 2 shows standard curves for CRP assay, as analyzed by using the antibody-antibody method (A), the  
20 antibody-PC method (B) and the PC-antibody method (C);

Fig. 3 shows the results of CRP assay in 100-fold diluted human serum obtained by using each of the above methods;

Fig. 4 shows the results of CRP assay in 100-fold  
25 diluted serum by the antibody-antibody method (A) and the antibody-PC method (B);

Fig. 5 shows the quantitative precipitation of CRP with  $\text{PC}_{58}$ -BSA;

Fig. 6 shows the comparison in CRP-binding activity between PC<sub>58</sub>- and PC<sub>γ</sub>-BSA;

Fig. 7 shows the inhibition of the PC-binding activity of CRP by water-soluble PC; and

5 Fig. 8 shows specific and non-specific CRP bindings according to the antibody-PC method (B).

#### DETAILED DESCRIPTION OF THE INVENTION

The present inventors have studied extensively various CRP assay methods. As a result of such studies,  
10 the inventors have found that proteins of the pentraxin family, such as amyloid A and amyloid P, are present in human serum and these proteins react with anti-CRP antibodies. It is suggested that one of the factors inhibiting the elevation of the assay sensitivity for the  
15 assay of CRP concentration by ELISA is the reactivity of the proteins with anti-CRP antibodies and that it is therefore very difficult to accurately determine CRP in, for example, human serum samples.

Focussing on the reactivity of CRP with C-  
20 polysaccharide existing in the capsule of Diplococcus pneumonia to bind to the phosphorylcholine group of the C-polysaccharide, the present inventors have determined the possibility of realizing highly sensitive assay of CRP concentration, by utilizing the specific binding of CRP to  
25 phosphorylcholine.

In accordance with the present invention, an assay is thus performed by a sandwich assay method using the specific binding of CRP to phosphorylcholine (PC). In a

preferred embodiment, PC is labeled and then used. In such a case, PC may be directly labeled or indirectly labeled by conjugating another molecule to PC, wherein said molecule mediates a labeling means. Additionally, PC  
5 may be labeled with a radioactive labeling means or with a non-radioactive labeling means. Further, these labeling means are not limited to currently available labeling means.

As a radioactive labeling means for labeling PC,  
10 conventional radioisotopes such as  $^{125}\text{I}$  and  $^{32}\text{P}$  can be used. As a non-radioactive labeling means for labeling PC, any of the following methods can be used: methods using enzymes, such as alkali phosphatase (AP), horse radish peroxidase,  $\beta$ -galactosidase and glucose oxidase; methods  
15 using luminescence from luciferin with luciferase; and methods using fluorescent dyes such as fluorescein and lanthanide. However, it should be noted that since the use of radioactive materials may not be preferable in conducting examination on site in clinical practice, the  
20 use of a non-radioactive labeling means is generally preferable. In one embodiment of the invention, lanthanum-series elements (lanthanides) are particularly used as fluorescent substances.

Lanthanides have been drawing attention in recent  
25 years as fluorescent probes for use in assaying biologically active compounds (for example, enzyme, lectin, receptor, and the like) and for histochemical research studies. Lanthanides emitting fluorescence, samarium



(Sm<sup>3+</sup>), europium (Eu<sup>3+</sup>), and terbium (Tb<sup>3+</sup>) are known [Saito, K., et al., (1998) Anal. Biochem., 258, 311-314; Elliot, C. T., et al., (1994) Analyst, 119, 2565-2569; Pettersson, K., et al., (1993) Clin. Chem., 39, 2084-2089; Rothlin, M. A., et al., (1993) Cancer, 71, 701-707; Samiotaki, M., et al., (1997) Anal. Biochem., 253, 156-161]. One of the advantages of utilizing fluorescence emitted from such lanthanides is that sensitivity at the same level as or at a level higher than that recovered by the methods utilizing radioisotopes can be obtained.

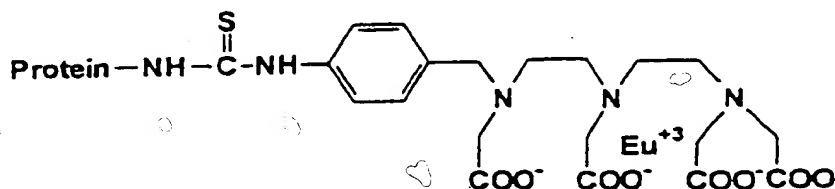
Any lanthanide which emits fluorescence can be utilized as fluorescent substance in labeling PC with lanthanides in the present invention. Among them, the use of Eu<sup>3+</sup> is preferable. Eu<sup>3+</sup> exhibits high sensitivity, has a long fluorescence life (far longer than the mean background fluorescence by FIA), and, in addition, the shelf life of Eu<sup>3+</sup> labeling reagents is far longer than that of RIA reagents.

The present invention will now be described herein below with reference to an example of the assay method of CRP concentration by a dissociation-enhanced lanthanide fluorescent immunoassay (DELFI) method [Guzaeva, T. V., et al., (1993) Immunology Lett. 35, 285-290] using lanthanides, particularly Eu<sup>3+</sup>.

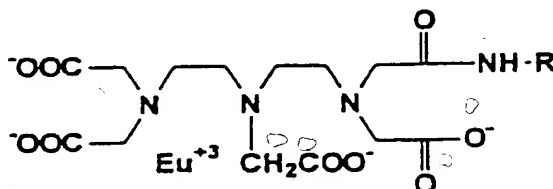
In order to label PC with Eu<sup>3+</sup>, the method described in the report of Saito, K., et al. (Saito, K., et al., (1998) Anal. Biochem. 258, 311-314) is used. According to this method, in order to label protein or other amino

compounds with  $\text{Eu}^{3+}$ , various chelating substances can be used, for example an EDTA phenylisothiocyanate derivative  $\text{N}^1$ -(p-isothiocyanatebenzyl)-diethylenetriamine- $\text{N}^1$ ,  $\text{N}^2$ ,  $\text{N}^3$ ,  $\text{N}^3$ -tetraacetic acid (DETATA-ITC) represented by the

5 following formula:

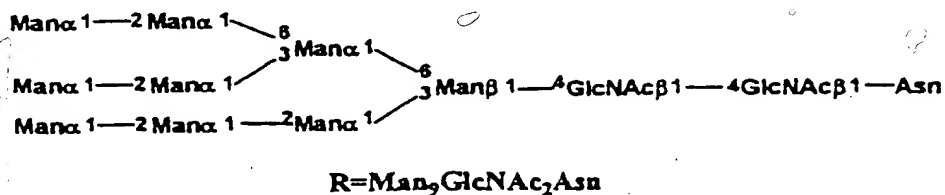


or diethylenetriamine- $\text{N}^1$ ,  $\text{N}^2$ ,  $\text{N}^3$ -pentaacetic acid (DTPA) represented by the following formula:



[in the formula, R represents amino acid or glycopeptides, for example asparagine-bound type sugar chain as

10 represented by the following formula:



] or dianhydride thereof (DTPAda). The protein can be labeled with  $\text{Eu}^{3+}$  by binding these chelating substances by

way of the functional groups thereof to a protein and the like.

In order to label PC ( $[(CH_3)_3NCH_2CH_2OPO_3H_2]^+$ ) with these chelating substances in accordance with the present invention, a protein having no influence on the reaction is first bound to PC to prepare a PC-protein complex. Once this is achieved, the chelating substances are bound to the protein. A protein may also be first labeled and then bound to PC. The protein is bound to PC by substituting the amino group of the protein with the PC group. Any compound which lacks an amino group can also be used provided that the compound can introduce substance chelating lanthanides.

It is required that the protein to be bound to PC is able to bind a single or multiple molecules of  $Eu^{3+}$ , and must have no influence on the reaction. Any protein which satisfies these conditions can be used, and they include, but are not limited to, any available protein, for example bovine serum albumin (BSA), keyhole limpet hemocyanine (KLH), ovalbumin (OVA) and rabbit serum albumin. More preferably, BSA or KLH are used. Most preferably, BSA is used. A person having ordinary skill in the art will readily understand that substances other than proteins are also applicable for the purpose under discussion.

Using chelating substances, PC was labeled with  $Eu^{3+}$  by using DTPA in accordance with the method reported above, namely, that of Kawasaki, N., and Lee, Y. C. (1997) Anal. Biochem. 250, 260-262.  $PC_{35}$ -BSA and  $PC_{58}$ -BSA, and BSA

respectively contain 7-, 6- and 4 molecules of  $\text{Eu}^{3+}$  per one molecule of the protein.  $\text{PC}_{35}$ -BSA as used herein means a conjugate where 35 PC molecules on average are bound per one BSA molecule;  $\text{PC}_{58}$ -BSA herein means a conjugate where  
5 58 PC molecules on average are bound per one BSA molecule. The quantity of the bound PC is almost equal to the quantity of the  $\text{Eu}^{3+}$  bound to IgG. When protein, for example anti-hCRP polyclonal rabbit IgG (molecular weight of 160 kDa) is labeled by a conventional method, 9 atoms  
10 of  $\text{Eu}^{3+}$  are bound per one molecule of the protein.

In accordance with the method of the present invention,  $\text{Eu}^{3+}$  for fluorometry is first dissociated from an initial chelating substance to form a complex, along with 1-( $\beta$ -naphthyl)-3,3,3-trifluoroacetone ( $\beta$ -NTA). The  
15 complex emits intense fluorescence in the presence of a surfactant (the fluorescence is referred to simply as  $\text{Eu}^{3+}$  fluorescence hereinafter). The duration of the attenuation of  $\text{Eu}^{3+}$  fluorescence is longer by  $10^4$ -fold, as compared with polystyrene and other plastics and glass  
20 materials. Thus, the time-resolved fluorescence of  $\text{Eu}^{3+}$  in a microtiter plate can be detected with a time-resolved fluorescence microtiter plate reader (Victor, Model 1420, Wallac Co., Gaithersburg, MD) (time-resolved fluorometry). Because  $\text{Eu}^{3+}$  fluorescence can be detected following  
25 irradiation with an excitation beam for a given period of time with subsequent sufficient attenuation being conducted for the fluorescence emitted from the immobilizing phase, any adverse influence of background

fluorescence can be eliminated.

Specific conditions for detecting fluorescence (in cps) from  $\text{Eu}^{3+}$  dissociated from the chelating substance bound to the protein may be determined appropriately. As one example,  $\text{Eu}^{3+}$  fluorescence is detected by means of single photon counting, using a xenon flash lamp (1,000 Hz) for a total counting time of 1 second, with a 50- $\mu\text{s}$  delay time and a 250- $\mu\text{s}$  counting time per flash ( $\lambda_{\text{ex}} = 340$  nm;  $\lambda_{\text{em}} = 615$  nm), after 15-min incubation in a 200  $\mu\text{l}$ /well enhancement solution [Hemmila, I., et al., (1984) Anal. Biochem. 137, 335-343] at ambient temperature. For CRP assay and PC-binding assay by the time-resolved fluorescence immunoassay (TR-FIA) method, the value of the CRP concentration in 20 mM EDTA-TBST was used as a background value.

When  $\text{Eu}^{3+}$  dissociation hardly occurs due to the intense binding of  $\text{Eu}^{3+}$  to the chelating substance, the labeled sample may be recovered from the plate, and then the sample may be burned, to modify all the organic materials therein into ashes containing only inorganic salts, to thereby enable assay of the  $\text{Eu}^{3+}$  concentration to be effected. In determining  $\text{Eu}^{3+}$  concentration by means of ash preparation, a method essentially established for determining organic phosphoric acid is used [Ames, B. N. and Dubin, D. T., (1960) J. Biol. Chem., 235, 769-758]. The ash sample is again dissolved by means of incubation in 1.5 ml of 0.1 M acetic acid at 55°C for 30 minutes. The  $\text{Eu}^{3+}$  fluorescence in an appropriate volume of the

dissolved sample (corresponding to 20 to 200 fmol  $\text{Eu}^{3+}$ ) is then able to be detected by the DELFIA method. Based on the results, the  $\text{Eu}^{3+}$  concentration can be assayed accurately even after ash preparation [Saito, K., et al.,  
5 (1998) Anal. Biochem. 258, 311-314].

Sandwich assay methods for determining CRP concentration in samples by using various labeling means are broadly grouped into an antibody-antibody method (A) as a sandwich assay using two species of antibodies and  
10 sandwich assay methods [(B) and (C)] using PC (see Fig. 1).

The method for determining CRP concentration by the antibody-antibody method (A) comprises the steps of:

- i) immobilizing an anti-CRP antibody on a plastic  
15 plate;
- ii) reacting a sample solution in which the CRP concentration is intended to be assayed, with the anti-CRP antibody; and
- iii) reacting a labeled anti-CRP antibody with the  
20 sample solution to thereby determine the CRP concentration in the sample [Fig. 1 (A)].

The inventors have nevertheless found that CRP present in samples obtained from humans, particularly serum CRP can not be accurately assayed by CRP assay using  
25 the antibody-antibody method (A). The reason is probably that in human serum amyloid A (hSAA) and P(hSAP) may react with anti-CRP antibodies. More specifically, hSAA and hSAP are fairly homologous to human CRP (66% and 70%,

respectively) [Agrawal, A., et al., (1994) in Acute Phase  
Proteins-Molecular Biology, Biochemistry and Clinical  
Applications-(Mackiewicz, A., et al., eds.), pp. 79-92,.  
CRC Press, London; Shrive, A. K., et al., (1996) Nature  
5 Struc. Biol. 3, 346-354; Kilpatrick, J., and Volanakis, J.  
(1991) Immunol. Res. 10, 43-53]. Accordingly, hSAA and  
hSAP may bind to the anti-CRP antibodies to be used for  
the assay. When used, anti-CRP polyclonal antibodies  
particularly involve difficulty in the elimination of the  
10 influence of the pentraxins contained in serum.

In accordance with the present invention, therefore,  
a sandwich assay method using only one anti-CRP antibody  
species and PC in place of the other antibody species is  
employed, so as to provide an assay method which is not  
15 subject to any of the aforementioned drawbacks. The  
invention encompasses two types of sandwich assay methods  
using PC, namely antibody-PC method (B) and PC-antibody  
method (C). The antibody-PC method (B) comprises the  
steps of:

20 i) immobilizing an anti-CRP antibody on a plastic  
plate;

ii) reacting a sample solution in which the CRP  
concentration is intended to be assayed, with the anti-CRP  
antibody; and

25 iii) reacting a labeled PC with the sample solution to  
determine the quantity of the label bound to the  
immobilizing phase to determine the CRP concentration in  
the sample [Fig. 1 (B)]. Alternatively, the PC-antibody

method (C) comprises the steps of:

i) immobilizing PC on an immobilizing phase such as a plastic plate;

ii) reacting a sample solution in which the CRP  
5 concentration is intended to be assayed with PC; and

iii) reacting an anti-CRP antibody with the sample solution to assay the quantity of the label bound to the immobilizing phase and thereby to determine the CRP concentration in the sample [Fig. 1 (C)].

10 In accordance with the present invention, reaction between CRP and PC is performed at least once by such a sandwich method. Because PC is not reactive with other pentraxin family members, unlike anti-CRP antibodies, the influence of undesirable reactions of PC with hSAA and  
15 hSAP, which is disadvantageous for the antibody-antibody method, can be prevented.

In accordance with the present invention, any of the sandwich assay methods using PC may be satisfactorily used. So as to enhance assay sensitivity, the antibody-PC  
20 method (B) is more preferably used. The PC-antibody method (C) has a drawback in that it causes a reduction in assay sensitivity, because serum lecithin blocks the PC-binding site of CRP in the presence of calcium at the reaction stage between the PC on the immobilizing phase  
25 and the CRP in a serum sample. While, according to the antibody-PC method (B), the inhibition of the reaction due to serum lecithin and calcium can be prevented because PC is able to interact with CRP after washing the



immobilizing phase after the CRP in a serum sample is bound to the anti-CRP antibodies bound to the immobilizing phase. After the anti-CRP antibodies on the immobilizing phase react with a serum sample, the resulting

5 immobilizing phase is preferably washed with an EDTA solution. By using the antibody-PC method (B), the minimum detection limit of a sample CRP concentration can be lowered to 420 fmol. It is readily understood that PC is used after being labeled for the antibody-PC method

10 (B).

#### Effects

The novel method for determining CRP of the present invention enables accurate assay of not only serum CRP concentration during an acute phase reaction such as in  
15 the occurrence of infection, inflammation and tissue damage, but also serum CRP concentration within a normal range. The novel method for determining CRP of the present invention further allows detection of serum CRP within a normal range with a high degree of sensitivity in  
20 a rapid fashion in clinical diagnosis. In addition, because the volume of a sample required for the assay is small (100  $\mu$ l per well), valuable serum samples can be saved and can thereby be subjected to the multiple assays thereby further improving accuracy.

#### 25 Examples

The materials used in the following examples are as follows.

Recombinant human C-reactive protein (rhCRP)

expressed in Escherichia coli and anti-hCRP polyclonal rabbit IgG were obtained from Oriental Yeast Co., Ltd. (Japan). Phosphorylcholine (PC) chloride (calcium salt), L- $\alpha$ -phosphatidylcholine (L- $\alpha$ -lecithin derived from egg yolk), potassium hydrogen phthalate, Triton X-100 and human AB-type female sera were obtained from Sigma Chem. Co. (St. Louis, MO). Rat serum was obtained from Pel-Freez Co. (Rogers, AR). Chelex 100 resin (sodium form) was obtained from Bio-Rad (Hercules, CA). N<sup>1</sup>-(p-  
10 isothiocyanatebenzyl)-diethylenetriamine-N<sup>1</sup>, N<sup>2</sup>, N<sup>3</sup>, N<sup>3</sup>-tetraacetic acid (DETATA-ITC) was obtained from Wallac (Gaithersburg, MD); and a 96-well microplate (cat. #21-377-204) was obtained from Fisher Scientific (Springfield, NJ). Europium nitrate, 1-( $\beta$ -naphthoyl)-3,3,3-  
15 trifluoroacetone, trioctylphosphine oxide (TOPO), diethylenetriamine-N<sup>1</sup>, N<sup>2</sup>, N<sup>3</sup>-pentaacetic acid (DTPA) and its dianhydride (DTPAda) were obtained from Aldrich Chem. Co. (Milwaukee, WI). Polyvinylbenzyl lactonoylamide (PVLA) was a gift from Dr. Kazukiyo Kobayashi (Nagoya  
20 Univ., Japan). As PC conjugates of PC<sub>7</sub>-, PC<sub>35</sub>- and PC<sub>58</sub>-BSA, and bovine serum albumin (BSA), those produced by the previously reported method [Stults, N., et al., (1987) Anal. Biochem. 161, 567-573] were used.

Example 1: Comparison of assayed CRP concentrations in sera, individually using the antibody-antibody method, the PC-antibody method and the antibody-PC method

#### Buffers and dissociation enhancement solution

Two types of buffers for use in CRP assay were i)

TBS (20 mM Tris-HCl, pH 7.6 containing 0.15 M NaCl and 10 mM CaCl<sub>2</sub>) and ii) TBST (TBS buffer containing 0.02% Tween 20).

The dissociation enhancement solution was composed of 15 µM β-NTA, 50 µM TOPO and 0.1% (w/v) Triton X-100 in 0.1 M acetic acid adjusted to pH 3.2 using potassium hydrogen phthalate [Hemmila, I., et al., (1984) Anal. Biochem. 137, 335-343].

#### Microtiter plate coating

The anti-CRP IgG antibody coated on the plate was increased in quantity, following an increase in the IgG concentration up to 5 µg/ml. Above this level, no increase in the coated quantity was observed. For the assay on microplates and the binding assay, thus, the antibody of 500 ng was left to stand in wells for coating.

Because it was difficult to immobilize PC-BSA without modification of the microtiter plate, PC-BSA was immobilized on the wells of a microtiter plate by using the PVLA method [Suzuki, N., et al., (1997) Anal. Biochem. 247, 412-416]. PVLA (2 mg) was suspended in 1 ml of water by ultrasonication; the resulting PVLA suspension of 40 µl was added to each well, for overnight incubation at 4°C. 1 mM NaIO<sub>4</sub> solution was added in 50 µl-portions to each well, and the plate was incubated in darkness at ambient temperature for one hour. After the plate was washed, the PC-BSA solution (100 ng protein in 40 µl of water) was added to each well, followed by mixing with 5 µl of 2% (v/v) borane-pyridine complex, for incubation at 37°C for

2 hours. The wells were blocked through incubation with 1M glycylglycine (40  $\mu$ l) at 37°C for 4 hours, and then followed by incubation with 200  $\mu$ l of TBS containing 2% (w/v) BSA at 4°C overnight. After the added solutions were removed at the individual stages of the following method, the wells were washed six times with 200  $\mu$ l of TBS.

To immobilize PC-BSA using the PVLA method, the level of coating was increased to 100 ng of the PC-BSA used per well, depending on the quantity of PC-BSA used. Thus, 100 ng of PC-BSA was routinely used for the PVLA method.

After preliminary tests were carried out, the level of Eu<sup>3+</sup>-labeled anti-CRP IgG (20 ng IgG, 1 pmol Eu<sup>3+</sup>/well) or PC<sub>35</sub>-BSA (20 ng BSA, 1.8 pmol Eu<sup>3+</sup>/well) was selected, so that the background count could be minimized.

#### CRP assay by DELFIA method

The principles of the three assay methods are shown in Fig. 1. Unless otherwise stated, the reagent solutions were discarded at the individual stages described, followed by washing of the wells six times with 200  $\mu$ l TBST. Eu<sup>3+</sup> fluorescence was finally detected by the DELFIA method previously reported [Guzaeva, T. V., et al., (1993) Immunol. Lett. 35, 285-290].

#### (A) Antibody-antibody method

100  $\mu$ l of TBS containing the anti-CRP antibody (500 ng) was added to each well, for overnight incubation at 4°C. Each well was blocked through incubation in 200  $\mu$ l

of 2% (w/v) BSA (in TBS) at ambient temperature for 2 hours; after discarding the BSA solution, the well was washed six times with 200  $\mu$ l of TBS. 100  $\mu$ l each of TBST samples containing variable quantities of CRP was applied to the wells coated with the antibody, followed by incubation at ambient temperature for 2 hours. After discarding the CRP solution, 100  $\mu$ l of a TBST buffer solution containing 20 ng of  $\text{Eu}^{3+}$ -labeled anti-CRP antibody (9  $\text{Eu}^{3+}$  per IgG) was added to the wells. Subsequently, the plate was incubated at ambient temperature for 2 hours, to detect  $\text{Eu}^{3+}$  fluorescence.

(B) Antibody-PC method

A microtiter plate coated with the anti-CRP antibody and blocked with BSA was prepared as described above. 100  $\mu$ l each of TBST containing CRP at variable quantities were left to stand in the wells, which were incubated at ambient temperature for 2 hours. After the CRP solution was discarded from the wells, 100  $\mu$ l of TBST containing  $\text{Eu}^{3+}$ -labeled PC-BSA was added to the wells, for incubation at ambient temperature for 2 hours. CRP treated with TBST containing 20 mM EDTA, and BSA labeled with  $\text{Eu}^{3+}$  were added to the coated wells, which were used as a background and as a standard.

(C) PC-antibody method

So as to immobilize  $\text{PC}_{58}$ - or  $\text{PC}_7$ -BSA solution (40  $\mu$ l containing 100  $\mu$ g protein and PC of about 120 pmol or 15 pmol) in water, the solution was left to stand in the PVLA-coated well. After the wells were coated with PC-BSA

and blocked with BSA, they were then washed six times with TBS (200 µl). 100 µl each of TBST containing various concentrations of CRP were introduced in the wells, for incubation at ambient temperature for 2 hours. After discarding the CRP solutions, the Eu<sup>3+</sup>-labeled anti-CRP antibody (20 ng in 100 µl of TBST) was added, and the plate was then incubated at ambient temperature for 2 hours.

#### Comparison of values of CRP concentration assayed

CRP assays by the antibody-antibody method (A) and the PC-antibody method (C) individually exerted two-phase standard curves (10 to 60 ng and 100 to 200 ng, respectively) (Fig. 2b). Alternatively, the results of the CRP assay by the antibody-PC method (B) were on a line within a range of 5 to 500 ng CRP/ml (Fig. 2a). The range was far wider than the ranges shown in Fig. 2b.

The antibody-antibody method (A) and the antibody-PC method (B) were not influenced by the presence of serum. rhCRP added to sera was not accurately determined by the PC-antibody method (C) (Fig. 3). Despite the presence or absence of BSA or PdC, rhCRP at known quantities in 100-fold diluted sera was effective in bringing about results expected from the antibody-antibody method (A) and the antibody-PC method (B). However, for the antibody-antibody method (A), the acceptable results with negligible interference were obtained only within a limited range (Fig. 4).

The DELFIA technique has enabled the development of

a highly sensitive CRP assay method, as regards CRP assay. The degree of sensitivity and accuracy of the antibody-PC method (B) using a plate coated with the anti-CRP antibody and PC-BSA labeled with  $\text{Eu}^{3+}$  are so high that 400 fmol  $\text{Eu}^{3+}$  (namely, 420 fmol CRP) can be detected. The specific binding can be recovered by removing a non-specific binding count from the total binding count (Fig. 8). The sensitivity corresponds to the range of 5 to 500 ng CRP/ml (Fig. 2) and is comparative to that of radioimmunoassay (8 to 800 ng CRP/ml) [Claus, D. R., et al., (1976) J. Lab. Clin. Med. 87, 120-128]. The standard deviation from triplet assays was less than 3%. Further, the linear range detectable by the invented method was far wider as compared with general ELISA methods [by ELISA methods, the range was 10 to 100 ng CRP/ml (Unten, S. K., and Hokama, Y. (1987) J. Clin. Lab. Anal. 1, 136-139)]. The antibody-PC method (B) directly uses PC-BSA labeled with  $\text{Eu}^{3+}$  in place of a secondary antibody, and is thus simpler. Owing to the assay sensitivity, CRP at a normal levels in serum can also be assayed even by using a serum sample in 10- to 100-fold dilution.

Example 2: PC-binding assays, individually using the antibody-antibody method, the PC-antibody method and the antibody-PC method.

#### 25 Buffers and dissociation enhancement solution

The two types of buffers and the dissociation enhancement solution used for PC-binding assay were prepared in the same manner as those used for CRP assay.

### Microtiter plate coating

The microtiter plate used for PC-binding assay was prepared by using anti-CRP IgG antibody or PC-BSA in the same manner as the plate used for CRP assay.

### 5      Quantitative precipitation of CRP

Quantitative precipitation reaction was carried out as follows: variable quantities of PC<sub>58</sub>-BSA in TBS was added to CRP (50 µg) in TBS to a final volume of 50 µl. After incubation at 37°C for one hour, the resulting  
10 mixture was left to stand at 4°C for 48 hours and then centrifuged (10,000 g x 5 minutes). The precipitate was washed three times with cold TBS and was then dissolved in 0.5 M NaOH (50 µl).

Protein concentration in the precipitate was  
15 determined on the basis of bicinchoninic acid (BCA) assay with a Pierce kit (Rockford, IL) by using rhCRP as a CRP standard or by using BSA as a PC-BSA standard [Smith, P. K., et al., (1985) Anal. Biochem. 193, 76-85]. When BSA is used as a standard for CRP assay, a coefficient of 0.84  
20 should be used so as to assay accurately CRP concentration by the BCA method. Phosphoric acid was measured by using phosphorylcholine as a standard by the organic phosphoric acid analysis method of Ames, et al. [Ames, B. N., and Dubin, D. T. (1960) J. Biol. Chem. 235, 765-775].

### 25      Interference by BSA, lecithin (PdC) and human sera

By using the aforementioned three methods, namely the antibody-antibody method (A), antibody-PC method (B) and PC-antibody method (C), CRP was assayed in a serum in



100-fold dilution with TBST. The rhCRP (10 to 40 ng/well) was added to the diluted serum, and the resulting mixture was left to stand in wells for assay. The influence of the presence of serum on these methods was compared using  
5 rhCRP (10 ng) as a standard. Similarly, TBST containing BSA (10 mg/ml) or PdC (1 mg/ml) was added to a solution containing variable quantities of rhCRP in order to examine the interference of said TBS containing TBST and PdC. As a blank test, CRP was diluted with TBST  
10 containing 20 mM EDTA.

PC-binding assay by DELFIA method

The three assay methods used for PC-binding assay were identical to the three methods used for CRP assay. And as with CRP assay,  $\text{Eu}^{3+}$  fluorescence was finally  
15 detected according to the DELFIA method.

PC-binding assay

CRP concentration in mol is expressed as the pentamer (molecular weight of 118,000).

(i) Precipitation method

20 The CRP activity to bind PC was assayed through quantitative precipitation by using 10 to 80  $\mu\text{g}$   $\text{PC}_{58}$ -BSA and 50  $\mu\text{g}$  CRP to determine the protein and phosphoric acid in the precipitate and assay the binding activity of CRP (Fig. 5). The precipitate reached a plateau at 20  $\mu\text{g}$  of  
25 the PC-BSA level, while 10 nmol phosphoric acid was observed in 70  $\mu\text{g}$  of the precipitate (as BSA).

(ii) Microtiter plate method

Using the antibody-PC method (B) and the PC-antibody

method (C), the PC-binding activity was assayed, using 20 ng of a  $\text{Eu}^{3+}$ -labeled probe, within a CRP range of 5 to 100 ng per well. In the case that PC-BSA or PdC was directly coated on plastic wells, no material coated (with PC-BSA or PdC) at a sufficiently high level for the assay of the binding activity of CRP was recovered, even when a  $\text{Eu}^{3+}$ -labeled probe was used (the data not shown). Thus, a PC-BSA immobilized plate was prepared by using the PVLA method.

10           When BSA (10 mg/ml) was present, the antibody-PC method (B) or the PC-antibody method (C) was never influenced; when 1 mM PdC (PdC concentration in serum) or diluted serum was present, the PC-antibody method (C) was influenced but the antibody-PC method (B) was not  
15           influenced (Fig. 3).

          It is surmised that the interference of the serum components in the PC-antibody method (B) may be due to the reaction of PdC mainly with the PC-binding site of CRP (Fig. 3). In fact, it is known that CRP precipitates  
20           together with lecithin in the presence of calcium [Hokama, Y., et al., (1974) Clin. Chem. Acta 50, 53-62]. So as to prevent such PdC interference, therefore, it was indicated that the antibody-PC method (B) which enables anti-CRP antibodies with no relation to the PC-binding activity to  
25           capture CRP from serum samples, was preferably used. Further, after a sample containing CRP was applied to the plate, additionally, the plate was preferably washed with an EDTA solution. This is intended to remove inhibitory

substances in serum from the PC-binding site (data not shown).

### Example 3: CRP activity to bind to PC

#### Preparation of Ca-free PC

5           Phosphorylcholine chloride (calcium salt) was suspended in water, together with 100 ml of Chelex 100 (sodium type); and the resulting suspension was agitated overnight at ambient temperature. The resin was filtered under aspiration. On the filter, the resin was then  
10 washed in water. Organic phosphoric acid in the resulting aqueous PC solution (sodium salt) was analyzed and freeze-dried into powder.

#### Inhibition of PC-binding activity by PC

          Various amounts of PC were incubated in a total 100  
15  $\mu$ l volume of each of TBST solutions containing 2, 5, 10, 50 and 100 ng CRP, at 37°C for 2 hours. The resulting mixtures were left to stand in wells immobilized with PC<sub>58</sub>-BSA, for incubation at ambient temperature for 2 hours. The CRP activity to bind to PC was assayed by using anti-  
20 CRP, as described above.

#### CRP activity to bind to immobilized PC-BSA

          The anti-CRP antibody (IgG) was bound at 5.6 f mol and 2.2 fmol per well to PC<sub>58</sub>-BSA-coated well and PC<sub>7</sub>-BSA-coated well, respectively (Fig. 6). The inhibition  
25 potency of PC against the CRP activity to bind to the PC-BSA was assayed at various amounts of CRP. When CRP was at 2, 5, 10, 20, 50 and 100 ng, the PC concentration at 50% inhibition (IC<sub>50</sub>) was 5, 11, 16, 17, 27 and 37  $\mu$ M

(Fig. 7) respectively, when the PC-antibody method (C) was used.

The antibody-PC method (B) has additional advantages. Most of current CRP assay methods singly  
5 depend on anti-hCRP antibody. Because human serum amyloid A (hSAA) and P(hSAP) are of the pentraxin family and with considerable homology to hCRP (66% and 70%, respectively) [Agrawal, A., et al., (1994) in Acute Phase Proteins-  
Molecular Biology, Biochemistry and Clinical Applications-  
10 (Machiewicz, A., et al., eds.), pp. 79-92, CRC Press, London; Shrive, A. K., et al., (1996) Nature Struc, Biol. 3, 346-354; Kilpatrick, J., and Volanakis, J. (1991) Immunol. Res. 10, 43-53], anti-hCRP antibodies possibly react with these pentraxins when the antibodies are  
15 polyclonal antibodies and used as primary antibodies for the assay. Meanwhile, only CRP is reactive with PC-BSA [Eriksen, N., et al., (1994) in Acute Phase Proteins-  
Molecular Biology, Biochemistry and Clinical Applications-  
(Machiewicz, A., et al., eds.), pp. 93-106, CRC Press,  
20 London]. According to the antibody-PC method (B), therefore, only CRP is assayed through specific reaction with PC-BSA labeled with  $\text{Eu}^{3+}$  during the final stage of the method.

At saturation precipitation when 20  $\mu\text{g}$   $\text{PC}_{58}$ -BSA was  
25 added, the precipitate contained 0.17 nmol  $\text{PC}_{58}$ -BSA (10 nmol PC in total) and 0.37 nmol CRP, and the chemical amount of  $\text{PC}_{58}$ -BSA bound to CRP was 1:2.2 (as the ratio of  $\text{PC}_{58}$ -BSA : CRP; Fig. 5). The PC-BSA immobilized by the

PVLA method was approximately calculated to be 5 to 6 fmol  
( $2.4 \times 10^5$  to  $2.6 \times 10^5$  cps) per well based on the  $\text{Eu}^{3+}$   
count according to the PC-antibody method (C), provided  
that the ratio of PC-BSA : CRP: anti-CRP IgG in the  
5 complex was 1 : 1: 1 (Fig. 6).

The PC-binding affinity of CRP was assayed by  
equilibrium dialysis at 15°C using radioactively labeled PC  
and capillary electrophoresis at 21°C. The results were  $K_d$   
= 6.3  $\mu\text{M}$  [Anderson, J. K., et al., (1978) Fed. Proc. 37,  
10 1495] and  $K_d$  = 17.7  $\mu\text{M}$  [Heegaard, N. H. H., and Robey, F.  
A. (1993) J. Immunol. Methods 166, 103-110]. Since PC  
inhibited CRP at 0.2, 0.4, 0.9, 1.7, 4.2 and 8.5 pM at 37°C  
by using the PC-antibody method (C), the  $\text{IC}_{50}$  of PC was 5,  
11, 16, 17, 27 and 37  $\mu\text{M}$  in the order, consequently (Fig.  
15 7). The ligand concentration at 50% inhibition is  
frequently used to approximately express a dissociation  
constant when both the receptor- and ligand concentrations  
are below 1% of the actual  $K_d$  [Chang, K.-J., et al., (1975)  
Biochem. Biophys. Acta 406, 294-303]. Although the amount  
20 of PC-BSA immobilized on the well is not known, the amount  
occupies only 0.2% of the reported  $K_d$  value, even if the  
total amount of PC-BSA is immobilized. Additionally, the  
CRP concentration is below 0.01% of the  $K_d$  value. Thus,  
the  $\text{IC}_{50}$  value can approximately express binding constant  
25 ( $K_d$ ). In such a case, the  $K_d$  value (5 to 37  $\mu\text{M}$ )  
approximately calculated in such a manner is consistent  
with the results of the equilibrium dialysis and the  
capillary electrophoresis (6.3 to 17.7  $\mu\text{M}$ ).